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Simultaneous Detection and Genotyping of Three Genomic Groups of *Borrelia burgdorferi* Sensu Lato in Dutch *Ixodes ricinus* Ticks by Characterization of the Amplified Intergenic Spacer Region between 5S and 23S rRNA Genes

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We developed a rapid and reliable method for the identification *Borrelia burgdorferi* sensu lato species in ticks. We used the DNA sequence polymorphism of the spacer region between 5S and 23S rRNA genes, which has been shown to be able to discriminate between eight genomic groups of *B. burgdorferi* sensu lato (D. Postic, M. Assous, P. A. D. Grimont, and G. Baranton, Int. J. Syst. Bacteriol. 44:743–752, 1994). Spacer DNA was amplified by PCR and was then hybridized to five membrane-bound oligonucleotides. The oligonucleotides were specific for *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, and group VS116. A probe which reacted with all genomic groups of *B. burgdorferi* sensu lato was also used. Ninety-six ticks collected in the field were destructed by bead beating, and the supernatant was used directly in a PCR. *B. burgdorferi* sensu lato DNA was detected in 6 of 57 adult ticks (11%) and 9 of 39 nymphs (23%). *B. garinii* was found in three nymphs and four adults, three nymphs carried *B. afzelii*, and one adult and one nymph carried group VS116. Double infections with *B. afzelii* and group VS116 were found in two nymphs and one adult. Thus, our method can simultaneously identify three genomic groups of *B. burgdorferi* sensu lato in ticks collected in the field. This technique provides new ways to study the association of genomic groups present in ticks and the risk of Lyme borreliosis.

Borrelia burgdorferi sensu lato, the causative agent of the zoonosis Lyme borreliosis (LB), is transmitted by ticks of the genus *Ixodes* (7). The onset of LB is often marked by the development of an erythema migrans rash at the site of the tick bite. The infection can persist for years and may affect the nervous system, cause arthritis, or result in a chronic cutaneous manifestation, acrodermatitis chronica atrophicans (30). *B. burgdorferi* sensu lato has been divided into three groups on the basis of DNA relatedness: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (4, 8). This division corresponds to the specific rRNA gene restriction patterns, protein electrophoresis patterns, multilocus enzyme electrophoresis patterns, and reactivities of monoclonal antibodies with outer surface protein A (Osp) or the reactivities of polyclonal antisera with OspC (4, 6, 31, 35). The genetic relatedness of *B. burgdorferi* sensu lato isolates has also been linked to the restriction fragment length polymorphism (RFLP) of the spacer region between the 5S (*rrf*) and 23S (*rrl*) rRNA genes (29). The 5S and 23S rRNA genes are tandemly duplicated in *B. burgdorferi* sensu lato, and this constellation has not been found in other members of the genus *Borrelia* or in other eubacteria (14, 29). Recently, the number of *B. burgdorferi* sensu lato genomic groups has been extended to eight by *Mse*I RFLP analysis of the intergenic spacer region between the 5S and 23S rRNA genes (22). Two of the newly identified groups, VS116 and PotiB2, comprise European isolates, whereas groups DN127 and 21123 include North American isolates. Finally, there is *Borrelia japonica*, which is isolated from Japanese *Ixodes ovatus* ticks (22).

There is strong evidence that the division in genomic groups has clinical relevance for European LB. Studies in Belgium, France, and Germany have presented indirect evidence of the association of *B. garinii* with neurological symptoms, the association of *B. burgdorferi* sensu stricto with arthritis, and the association of *B. afzelii* with acrodermatitis chronica atrophicans (1, 3, 11). In a study of patients with LB in The Netherlands, Van Dam et al. (32) identified 56 of 57 spirochetal isolates from patients with erythema migrans or acrodermatitis chronica atrophicans as *B. afzelii* and 9 of 10 *Borrelia* isolates from patients with disseminated LB as *B. garinii* (32). Because of this apparent association between genomic groups and the clinical symptoms of LB, knowledge of the presence of *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto in ticks may give important information on the risk of LB in tick-inhabited areas. Identification of the genomic group by serotyping with monoclonal antibodies or RFLP analysis of rRNA genes requires isolation of the spirochete, a cumbersome and time-consuming procedure (4, 35). Typing methods which use PCR to amplify species-specific polymorphic DNA sequences are advantageous in this respect, because isolation of the organism can be omitted. Recently, three methods of differentiating *B. burgdorferi* sensu lato into genomic groups have been described: by PCR with genomic group-specific primers, analysis of the PCR product by DNA sequencing, or RFLP analysis (10, 12, 19). In the study described here, we used the spacer region between the 5S and 23S rRNA genes (rDNA) of *B. burgdorferi*

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TABLE 1. *B. burgdorferi* sensu lato isolates used in PCR and RLB

Isolate	Genomic group of *B. burgdorferi* sensu lato by RLB	Source	Origin	Supplier
20047	*B. garinii*	Tick	France	D. Postic
A39S	*B. afzelii*	Skin	The Netherlands	A. Van Dam
ACA-1	*B. afzelii*	Skin	Sweden	E. Åsbrink
AR-1	*B. garinii*	Tick	The Netherlands	RIVM[a]
AR-2	Group VS116	Tick	The Netherlands	RIVM
B31	*B. burgdorferi* sensu stricto	Tick	New York State	ATCC 35210[b]
BO23	*B. afzelii*	Skin	Germany	U. Göbel
DK-1	*B. afzelii*	Skin	Denmark	K. Hansen
ECM1	*B. afzelii*	Skin	Germany	U. Göbel
G1	*B. garinii*	CSF[c]	Germany	R. Marconi
HB-4	*B. burgdorferi* sensu stricto	Blood	New York State	S. Cutler
M19	Group VS116	Tick	The Netherlands	M. Nohlmans
N34	*B. garinii*	Tick	Germany	R. Ackermann
PKo	*B. afzelii*	Skin	Germany	V. Preac-Mursic

[a] RIVM, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.
[b] ATCC, American Type Culture Collection.
[c] CSF, cerebrospinal fluid.

sensu lato as the target for a nested PCR and determined the genomic group by hybridization of the PCR product to multiple genomic group-specific oligonucleotide probes immobilized on a membrane. This system, designated reverse line blotting (RLB), has other additional advantages: DNA sequencing of the PCR product is not required, small amounts of PCR product can be typed, different genomic groups can be detected concurrently, and coinfections of different genomic groups of *B. burgdorferi* sensu lato can be distinguished. Coinfections with two or more genomic groups of *B. burgdorferi* sensu lato have been found in ticks and patients with LB (10, 12, 18, 33).

A previous survey showed that between 13 and 43% of ticks collected on the Dutch island of Ameland contained *Borrelia* spp., and we isolated representatives of *B. garinii* and group VS116 from these ticks (24, 27). In the study described here, we used PCR and RLB to identify the presence of genomic groups of *B. burgdorferi* sensu lato in ticks collected in the field. We show that three genomic groups are present in 96 *Ixodes ricinus* ticks collected on the Dutch island of Ameland and that double infections with strains from two genomic groups occur among these ticks.

MATERIALS AND METHODS

Study area and tick collections. In May 1994, ticks were collected by blanket-dragging in the woodlands on the island of Ameland in The Netherlands (53°27′N, 5°45′E). *I. ricinus* ticks collected from Ameland were maintained in the laboratory for four generations at 18°C with 100% relative humidity and a photoperiod of 14 h of light and 10 h of darkness. Lysates of laboratory-reared ticks were negative for *Borrelia* DNA by PCR. Ticks that were used for PCR were stored in 70% ethanol at −20°C. Although the ticks investigated in the present study originated from the same batch collected in May 1994, different individual ticks were used either for indirect fluorescence antibody assay (IFA) or for PCR.

***Borrelia* strains.** The genomic groups and the biological and geographical origins of the *B. burgdorferi* sensu lato strains used in the study are given in Table 1.. The nomenclature for the *B. burgdorferi* sensu lato species was adopted from that of Postic et al. (22). For use in PCR and RLB, *B. burgdorferi* sensu lato strains were cultured in BSKII medium at 34°C, and DNA was isolated as described previously (5, 36). DNA prepared from *Borrelia hermsii* and *Borrelia anserina* was a gift from A. P. van Dam (Academic Medical Center, Amsterdam, The Netherlands), and *Treponema pallidum* subsp. *pallidum* cells originated from our own bacterial strain collection.

Detection of *Borrelia* spp. in ticks by immunofluorescence. Ticks were screened for the presence of *B. burgdorferi* sensu lato by using an IFA as described previously (24).

Preparation of tick samples for PCR. Ticks were processed for PCR by mechanical destruction (9). Fifty-seven adult and 39 nymphal *I. ricinus* ticks were air dried on paper and cut in half lengthwise. The two halves were transported to a reaction vessel (1.7 ml; Multi Technology Inc., Salt Lake City, Utah) which contained 200 μl of Saiki buffer (Perkin-Elmer, Gouda, The Netherlands [26]) supplemented with 2.5 mM $MgCl_2$ and 50 μl of zirconium beads of 0.5 and 0.1 mm, respectively, for the two halves (Biospecs, Bartelsvilles, Okla.). The suspension was vortexed on a Mini bead beater (Biospecs) for 3 min at a speed of 3,000 rpm and chilled on ice. After 2 min of centrifugation, the supernatant was stored at −20°C until it was used for PCR.

Detection of *B. burgdorferi* sensu lato DNA by PCR. All tick samples and DNAs of *B. burgdorferi* sensu lato isolates were tested in duplicate by nested PCR. *Taq* DNA polymerase (Amplitaq), primers (Table 2), and buffers were obtained from Perkin-Elmer. The first PCR was performed in a reaction volume of 25 μl containing 0.625 U of *Taq* DNA polymerase, Saiki buffer supplemented with 2.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate (dNTP), 5 pmol each of primers 23SN1 and 23SC (Table 2), and 5 μl of the supernatant of the processed tick sample. Finally, the mixture was sealed with 50 μl of paraffin oil. PCR tubes were briefly vortexed and centrifuged and were then transferred to a thermal cycler (Hybaid Omnigene TR3 SM2; Biozym, Landgraaf, The Nether-

TABLE 2. Oligonucleotide sequences of primers and probes used in PCR and RLB for detection and identification of *B. burgdorferi* sensu lato genomic groups

Designation	Target specificity	Nucleotide sequence	Position on 5S-23S intergenic spacer region
Primers for first PCR[a]			
23SN1	*B. burgdorferi* sensu lato	5′-ACCATAGACTCTTATTACTTTGAC	469–446
23SC1	*B. burgdorferi* sensu lato	5′-TAAGCTGACTAATACTAATTACCC	92–115
Primers for second PCR[a]			
23SN2	*B. burgdorferi* sensu lato	5′-ACCATAGACTCTTATTACTTTGACCA	469–444
5SCB	*B. burgdorferi* sensu lato	5′-biotin-GAGAGTAGGTTATTGCCAGGG	243–263
Probes for RLB[b]			
S1	*B. burgdorferi* sensu lato	5′-a-CTTTGACCATATTTTTATCTTCCA[c]	453–430
Ss	*B. burgdorferi* sensu stricto	5′-a-AACACCAATATTTAAAAAACATAA	322–299
Ga	*B. garinii*	5′-a-AACATGAACATCTAAAAACATAAA	322–298
Af	*B. afzelii*	5′-a-AACATTTAAAAAATAAATTCAAGG	305–278
VS116	VS116	5′-a-CATTAAAAAAATATAAAAAATAAATTTAAGG	303–278

[a] The positions and sequences of the primers are derived from Schwartz et al. (29).
[b] Probe positions are derived from Schwartz et al. (29), and sequences were obtained from Postic et al. (22). The nucleotide sequence and orientation of the probe are complementary and inverse to those for the 5SCB primer.
[c] a, aminolink spacer.

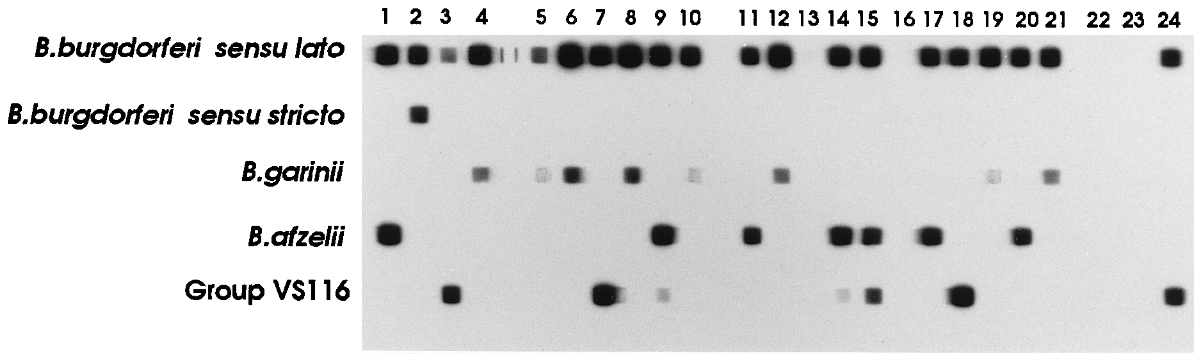

FIG. 1. Genotyping of *Borrelia* isolates and simultaneous detection and genotyping of genomic groups of *B. burgdorferi* sensu lato in tick samples. The indicated *Borrelia* isolates are in the following lanes: 1, A39S; 2, B31; 3, AR-2; 4, AR-1; 22, *B. hermsii*; 23, *B. anserina*; 24, group M19. The indicated ticks are in the following lanes: 5, 6, 8, and 10, adults infected with *B. garinii*; 7, adult infected with group VS116; 9, adult infected with group VS116 and *B. afzelii*; 11, 17, and 20, nymphs infected with *B. afzelii*; 12, 19, and 21, nymphs infected with *B. garinii*; 14 and 15, nymphs infected with *B. afzelii* and group VS116; 18, nymph infected with group VS116; 13 and 16, nymphs negative by PCR.

lands). The program for the first PCR consisted of an initial denaturation (1 min 94°C) and then 25 rounds of temperature cycling (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min). For the second PCR, the tubes were transported to a safety cabinet. A 5-μl reaction mixture containing 0.625 U of *Taq* DNA polymerase, Saiki buffer supplemented with 2.5 mM $MgCl_2$, 1 mM (each) dNTP, and 5 pmol of biotin-labeled primer 5SCB and of primer 23SN2 was added to each tube. PCR tubes were briefly vortexed and centrifuged and were then transferred to a thermal cycler, and after a denaturation step (1 min at 94°C), 40 rounds of temperature cycling (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) were performed. Contamination of tick samples with *B. burgdorferi* sensu lato DNA was monitored by using negative control samples (without tick sample) which had been subjected to identical procedures. The product of the first PCR had a size of approximately 380 bp, and DNA amplification by the second PCR resulted in a DNA molecule of 225 bp. To ascertain that amplification had taken place, 5 μl of the PCR mixture was analyzed on ethidium bromide-stained 2% agarose gels in a Tris-borate-EDTA (pH 8.2) buffer. All PCR samples which showed bands, albeit faint, bands of an erroneous size, or a smear were tested by RLB. Samples from ticks which were negative by PCR were spiked with 100 fg of B31 DNA (approximately 20 genome equivalents) to determine whether PCR was inhibited by tick components. The specificity of the nested PCR was determined with boiled *T. pallidum* (5×10^3 cells) and 25 ng of *B. hermsii* or *B. anserina* DNA. The sensitivity of the nested PCR was determined with DNAs from strains B31 (*B. burgdorferi* sensu stricto), A39S (*B. afzelii*), AR-1 (*B. garinii*), and AR-2 (group VS116) at concentrations of 0.1 to 100 fg of DNA per PCR sample.

Identification of genomic groups of *B. burgdorferi* sensu lato by RLB. RLB is a modification of the reverse dot blot, and probes are applied to the membrane as lines instead of dots (25). In one assay, the reactivity of 45 PCR products with up to 45 different probes can be determined (16). After amplification of DNA by PCR, the product was hybridized to four genomic group-specific oligonucleotides (Table 2) (*B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, and group VS116) and one probe which reacts with all genomic groups of *B. burgdorferi* sensu lato (Table 2); the latter probe served as a control on hybridization procedures. The probes were covalently linked to an activated Biodyne C membrane (Pall Europe Ltd., Portsmouth, United Kingdom) by the 5′ aminolink group. For this purpose, the membrane was activated by incubation for 15 min in 16% 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (Merck), rinsed with water, and placed in a miniblotter system (Immunetics, Cambridge, Mass.). Five slots were filled with 150 μl of an oligonucleotide suspension, which consisted of 12.5 to 100 pmol of probe (Perkin-Elmer) in 500 mM $NaHCO_3$ (pH 8.4). After 1 min of incubation, excess solution was aspirated, and the blot was removed from the miniblotter and inactivated by incubation with 100 mM NaOH for 10 min. After a rinse with water, the blot was incubated at 56°C in 2× SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 7.2])–0.1% sodium dodecyl sulfate (SDS) for 10 min, and after a rinse in 2× SSPE, the blot was stored at 4°C or was used immediately. The filter was again placed in the miniblotter, but the orientation was rotated 90° to the previous position, resulting in a perpendicular position of the slots on the lines which contained the oligonucleotides. The slots were filled with 150 μl of heat-denatured PCR products (10 μl of the PCR mixture suspended in 140 μl of 2× SSPE–0.1% SDS, boiled for 10 min, and chilled on ice) and incubated for 60 min at 45°C. The slots were aspirated, and the blot was removed from the miniblotter and washed twice for 10 min at 40°C with 2× SSPE–0.5% SDS. Ten milliliters of streptavidin-peroxidase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany) diluted 1:4,000 in 2× SSPE–0.5% SDS was added to the blot; this was followed by an incubation at 40°C for 30 min. Subsequently, the blot was rinsed twice with 2× SSPE–0.5% SDS at 40°C. Finally, the blot was briefly rinsed twice with 2× SSPE. Bound streptavidin-labeled PCR product was detected by chemiluminescence, which was performed with the ECL detection system (Amersham International plc, Den Bosch, The Netherlands), and visualized by exposure of the blot to an X-ray film (Hyperfilm; Amersham). PCR-amplified DNAs from *B. hermsii*, *B. anserina*, or *B. burgdorferi* sensu lato isolates were used to determine the specificity of the RLB. To determine the ability of the RLB to detect a mixture of two genomic groups simultaneously, different amounts of *B. afzelii* A39S DNA (500, 100, 50, 10, and 5 fg) were mixed with 500 fg of *B. garinii* AR-1 DNA. The DNA in the mixture was amplified by nested PCR and was hybridized to genomic group-specific probes in RLB.

Statistical analysis. The chi-square test of the package GraphPad Instat (version 1.14; GraphPad Software, San Diego, Calif.) was used to compare *B. burgdorferi* sensu lato infection rates in ticks obtained by IFA and PCR. A *P* value of ≤0.05 was considered significant.

RESULTS

Specificity and sensitivity of PCR and RLB. The sensitivity of the nested PCR was tested with a range of *B. burgdorferi* sensu lato DNA concentrations diluted in water and pooled tick lysate. *I. ricinus* ticks, which were maintained in the laboratory and which were free of *Borrelia* spp., were used as the source for a pool of tick lysates. In water, 5 fg of *Borrelia* DNA (from strains B31, AR-1, or A39S) was detected, and in tick lysates the detection limit of the nested PCR was 10 fg of DNA. The DNAs of *T. pallidum* and *B. anserina* were not amplified in the nested PCR, and amplification of DNA from *B. hermsii* yielded a fragment with a size of 500 bp (data not shown).

Fourteen *B. burgdorferi* sensu lato strains were tested by PCR and RLB, and the correct genomic group of all strains was identified from amplified 5S-23S rDNA sequences (Table 1). The reactivities of PCR samples from strains B31, A39S, AR-1, AR-2, and M19 in RLB are shown in Fig. 1. The PCR product of isolate M19 reacted with the probe for group VS116 (Fig. 1, lane 24). PCR products from *B. hermsii* or *B. anserina* were negative in the hybridization assay (Fig. 1, lanes 22 and 23). RLB could distinguish between the presence of *B. afzelii* DNA and *B. garinii* DNA in mixtures, which had been amplified by PCR, when 50 to 500 fg of *B. garinii* DNA was added to 500 fg of *B. afzelii* DNA (data not shown). Therefore, the presence of two genomic groups in a sample can be detected by PCR and RLB if the ratio between genomic groups does not exceed 1:10.

Detection and identification of *B. burgdorferi* sensu lato in ticks by PCR and RLB. A total of 96 ticks were investigated by PCR for the presence of *B. burgdorferi* sensu lato. Six of 57 (11%) adult ticks and 9 of 39 nymphs (23%) were positive for

TABLE 3. *B. burgdorferi* sensu lato infection rate in *I. ricinus* ticks collected on the Dutch North Sea island of Ameland as determined by IFA, PCR, and RLB

Tick stage	Infection rate by:				Genomic group by RLB[a]				
	IFA		PCR						
	No. tested	No. (%) positive	No. tested	No. (%) positive	Ss	Ga	Af	VS116	Af + VS116
Nymphs	120	23 (19)	39	9 (23)	0	3	3	1	2
Adults	90	18 (20)	57	6 (11)	0	4	0	1	1

[a] Ss, *B. burgdorferi* sensu stricto; Ga, *B. garinii*; Af, *B. afzelii*; VS116, group VS116; Af + VS116, infection of *B. afzelii* and group VS116.

B. burgdorferi sensu lato (Table 3). The discrepancy in the *Borrelia* infection rate for nymphs and adults was not statistically significant ($P = 0.96$). Tick lysates, which were negative for *B. burgdorferi* sensu lato by PCR ($n = 81$), were spiked with strain B31 DNA, and subsequently, *Borrelia* DNA was detected in all samples (data not shown). This finding indicated that PCR was not strongly inhibited by tick components because approximately 20 copies of the *Borrelia* genome could be detected in these tick samples. The analysis of 15 positive PCR samples in RLB is shown in Fig. 1. Among the samples of adult ticks, *B. garinii* was present in four samples (lanes 5, 6, 8, and 10), group VS116 was present in one sample (lane 7), and a double infection of group VS116 and *B. afzelii* was found in one sample (lane 9). Among the samples of nymphs, three samples contained *B. garinii* (lanes 12, 19, and 21), three samples contained *B. afzelii* (lanes 11, 17, and 20), and one sample reacted with the VS116 probe (lane 18). The two remaining samples contained *B. afzelii* and group VS116 (lanes 14 and 15). The overall results are summarized in Table 3.

Detection of *B. burgdorferi* sensu lato in ticks by IFA. *B. burgdorferi* sensu lato infection rates in adult and nymphal *I. ricinus* ticks collected on Ameland in 1994 were 20 and 19%, respectively (Table 3). Of the adult ticks, 9 of 42 (21%) females and 9 of 48 (19%) males contained *Borrelia* spirochetes; this difference was not statistically significant ($P = 0.96$). The observed discrepancies between the *B. burgdorferi* sensu lato infection rates determined by IFA or PCR for adult ticks ($P = 0.20$) or nymphs ($P = 0.76$) were not statistically significant. Infection rates for nymphs (20%; $P = 0.74$) or adults (24%; $P = 0.69$) were also not significantly different from those found in the survey performed in 1993 (24).

DISCUSSION

Our aim was to develop a method for the detection and identification of genomic groups of *B. burgdorferi* sensu lato isolates in ticks which does not require the prior isolation of *Borrelia* isolates. This would allow a more accurate description of the predominance of various genomic groups of *B. burgdorferi* sensu lato in their natural habitat. Such information is needed to more precisely define areas where individuals would be at high risk of contracting systemic LB such as neuroborreliosis or Lyme arthritis.

We used the 5S-23S rDNA intergenic spacer region as the target for nested PCR and hybridization. Previously, the 16S-23S rDNA region and the 23S-23S rDNA region have been used for a PCR to detect *B. burgdorferi* sensu stricto DNA in ticks and patient biopsy specimens, respectively (19, 28). By this PCR it was possible to detect 10 fg of *B. burgdorferi* sensu lato DNA diluted in tick lysate, which corresponds to approximately two organisms per sample. The PCR was shown to be species specific, and in RLB, the anticipated genomic group was identified from the PCR product of each *B. burgdorferi* sensu lato isolate ($n = 14$).

B. burgdorferi sensu lato DNA was amplified by PCR in 16% of ticks collected on Ameland in 1994. Complete inhibition of PCR by tick components has been found for *Amblyomma americanum* ticks (13). Therefore, PCR-negative ticks were spiked, and we established that approximately 20 copies of the *Borrelia* genome could be detected in these ticks; however, this does not rule out the possibility that these ticks contained lower numbers of *Borrelia* spirochetes. In a different group of ticks from the same batch, the presence of *Borrelia* spirochetes was analyzed by IFA. Infection rates for male and female ticks differed only marginally. A slightly higher percentage of infected nymphs was detected by PCR than by IFA, whereas the infection rate in adults was higher by IFA than by PCR. These differences, however, were not statistically significant and may have been due to a selection of individuals for each group.

Hybridization of 15 PCR-positive samples from ticks by RLB revealed three genomic groups of *B. burgdorferi* sensu lato: *B. afzelii*, *B. garinii*, and group VS116. These findings are in accordance with those from other studies performed in The Netherlands and Europe. *B. garinii* and *B. afzelii* are found in ticks and patients with LB throughout Europe, and both *Borrelia* species are also present in Dutch ticks (2, 20, 22, 32, 34, 35). Until now, only four *Borrelia* strains isolated from ticks collected in England, Switzerland, The Netherlands, and Japan have been classified as genomic group VS116 (2, 21, 22, 23, 27). *Borrelia* strains which belong to group M19, which is closely related to *B. garinii*, have been isolated from ticks collected throughout The Netherlands (20). In the present study, we show that group VS116 is found among ticks on Ameland and that strain M19 belongs to genomic group VS116 (Fig. 1). Thus, *B. burgdorferi* group VS116 is well established among Dutch ticks. The relevance of this genomic group for clinical LB remains to be investigated. Until now, patient isolates belonging to group VS116 have not been described. Kuiper et al. (17) reported a seropositivity rate of 20% among healthy Dutch forestry workers. Possibly, the majority of infections with *B. burgdorferi* group VS116 result in asymptomatic seropositivity. To examine this hypothesis, urine samples from asymptomatic seropositive individuals, which may contain *B. burgdorferi* sensu lato DNA, could be investigated by PCR (15).

Double infections with *B. afzelii* and group VS116 were found in two nymphs and one adult tick (20% of PCR-positive ticks). Cross-reactivity between PCR products is unlikely because the probes for VS116 and *B. afzelii* differ in 8 of 22 nucleotides, and the reference strains used in the present study did not display such cross-hybridization (Table 2 and Fig. 1). Thus, two different genomic groups were present in these ticks. Previous studies showed that double infections occur in European ticks. *B. burgdorferi* sensu lato strains with two different OspA genotypes have been isolated from 36% of infected ticks in Switzerland (18). Recently, sequencing of OspA DNA amplified from ticks collected in the field revealed one double infection among 40 PCR-positive ticks, which is considerably lower than our rate of double infection (12). This variation might be explained by differences in the tick populations studied or differences in study methodology. The typing method described in the latter study could not detect both genomic groups of *B. burgdorferi* sensu lato if the ratio of a mixture exceeded 1:5, whereas our method could distinguish two genomic groups at a ratio of 1:10. In one study (10), the simultaneous presence of *B. burgdorferi* sensu stricto, *B. afzelii*, or *B. garinii* was found in 8 of 18 patients with neuroborreliosis, and *B. garinii* was detected in all of these samples, consistent with the association of *B. garinii* with neuroborreliosis. Only these three genomic groups could be identified; therefore, further investigation of coinfections, including the involvement of newly recognized genomic groups such as VS116, is needed.

In conclusion, amplification and hybridization of the 5S-23S rDNA intergenic spacer region to multiple genomic group-specific oligonucleotides provide an accurate and rapid method of determining the presence of all genomic groups of *B. burgdorferi* sensu lato in ticks collected in the field.

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